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ANALYSIS OF SAXITOXIN FROM URINE USING DYNAMIC FAB/MS

FINAL REPORT

CHESTER J. MIROCHA
YOUNG BAE KIM
UROOJ MIRZA
WEIPING XIE
HAMED K. ABBAS

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Chester J. Mirocha
Weiping Xie
Young Bae Kim
Hamed K. Abbas
Urooj Mirza

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13. ABSTRACT *(Maximum 200 words)*

An improved method of saxitoxin analysis in urine using continuous Flow Fast Atom Bombardment Mass Spectrometry (CF/FAB/MS) was developed. Parameters studied were matrix composition, matrix flow rate, temperature of probe tip, probe tip design and sample extraction. Optimal detection was obtained using the following matrix composition: 5% glycerol, 0.5% acetic acid, 0.025% sodium dodecylsulfate, 0.1% polyethylene glycol (PEG) 400 and 0.5% PEG 300; probe tip temperature: (~55C); flow rate: 5 or 8 µl per min; probe tip: Olson-Hogge design. The STX standard was detected at 200 picograms with signal-to-noise ratio of 11. The percent recovery of saxitoxin from human urine after cleanup on a weak cation exchange (CBA Bond-Elut Analytichem Inter. Varian, Anaheim, CA) column was 75%.

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This project was instrumental in providing the opportunity to maintain state-of-the-art mass spectrometer analytical equipment for the analyses of toxins important to the national defense. It has also provided training for students in the area of analyses of naturally occurring toxins.

ANALYSIS OF SAXITOXIN IN URINE BY CONTINUOUS FLOW FAST ATOM BOMBARDMENT MASS SPECTROMETRY

Chester J. Mirocha*, Wonjo Cheong , Urooj Mirza and Young Bae Kim

Department of Plant Pathology, University of Minnesota, St. Paul MN 55108, USA.

ABSTRACT

An improved method of saxitoxin analysis in urine using Continuous Flow Fast Atom Bombardment Mass Spectrometry (CF/FAB/MS) was developed. Parameters studied were matrix composition, matrix flow, temperature of probe tip, probe tip design and sample extraction. Optimal detection was obtained using the following matrix composition: 5% glycerol, 0.5% acetic acid, 0.025% sodium dodecylsulfate, 0.1% polyethylene glycol (PEG) 400 and 0.5% PEG 300; probe tip temperature:(~55 C) ; flow rate: 5 or 8 ul per min.; probe tip: Olson-Hogge design. The STX standard was detected at 200 picograms with signal-to-noise ratio of 11. The percent recovery of saxitoxin from human urine after cleanup on a weak cation exchange (CBA Bond-Elut Analytichem Inter. Varian, Anaheim, CA) column was 75%

INTRODUCTION

Saxitoxin was first isolated and described from toxic Alaska Butter Clams known by the Latin binomial **Saxidomus giganteus** (1). The Paralytic Shellfish Poisons (PSP) include saxitoxin (STX) which is one of the most serious marine toxins known (2). When ingested by humans, it can cause paralysis often terminating in death. **Protogonyaulax** (formerly **Gonyaulax**) **catenella** produces STX and grows to a high density under favorable environmental conditions. The organism is ingested by bivalves which in turn may poison humans when consumed (3).

Numerous studies have been made on the occurrence, chemistry, structure and toxicity of STX and related shellfish poisons (1, 4-15). Schantz et al. (4) first determined the correct structure of STX. Schantz (6) characterized STX as a basic substance forming salts with mineral acids; the hydrochloride is hygroscopic, a white solid crystal, very soluble in water, sparingly soluble in methanol and ethanol and insoluble in lipid solvents. Two basic guanidine groups make up the molecule, one with a pKa of 8.3 and the other 11.5. Tanino (15) first reported on the total synthesis of STX.

Various analytical methods have been reported (16-27) including a well established HPLC separation (18-20, 22, 23) followed by postcolumn oxidation and detection by fluorescence.

Sullivan et al.(18) reported detection of 100 pg with this method. Wright et al. (27) reported on the resolution of derivatized STX by capillary zone electrophoresis (CZE) followed by laser fluorescence detection (50 pg).

Development of Fast Atom Bombardment (FAB) enabled analysis of STX by mass spectrometry (16, 17) although quantitative determination has not been reported. However, Quilliam et al. (26) reported an ion spray (IS/MS) mass spectrum (Sciex Taga 6000) and determined a detection limit of 30 pg by selected ion recording (SIR). Their study was carried out by open loop flow injection of pure STX standard. Thibault et al. (40) reported on CZE resolution of STX (extract from mussels) combined with IS/MS and a detection limit of 15 pg. CZE is a most effective method of resolving STX in picogram quantities.

The use of continuous flow FAB (CF/FAB) provides some significant advantages over conventional static probe FAB including increased sensitivity and lower detection limit due to a decrease in chemical noise and ion suppression effects (28-32). Moreover, it provides a means for rapid analysis of multiple samples. At present we are not aware of any method that has been reported utilizing HPLC/CF/FAB for the resolution and detection of PSP.

Our ultimate objective is to develop an analytical method based on CF/FAB/MS by which STX can be quantitatively detected in urine in the mid picogram range. We have focused on making

improvements in CF/FAB to achieve this goal.

In this paper, we wish to report on results of experiments in selection of a sensitive FAB matrix, probe tip design (flat, oval , round and fritted), probe tip temperature to obtain best sensitivity and peak shape, choice of surfactant in the FAB matrix, matrix flow rate, detection limit of STX as well as preliminary data on extraction and recovery of STX from urine.

EXPERIMENTAL

CF/FAB/MS was done on a VG 7070EQ instrument with a FAB source, CF/FAB probe and Xenon gun from Fisons (VG Analytical Ltd), Manchester, England. A Shimadzu (Shimadzu Inc. Kyoto, Japan) LC-600 dual piston pump (capable of delivering 1 ul/min) was used with 50 um i.d. fused silica (deactivated) 1 meter capillary columns (Scientific Glass Engr. Inc., Victoria, Australia). Injections (open loop infusion) were made with a Rheodyne (Cotati, California, USA) injector with 200 nl or 500 nl rotor or a Valco (Valco Instr. Inc. Houston, TX) 60 nl or 200 nl injector. Solvents used were the best available grade from Aldrich Chemical Inc., Milwaukee, WI. Saxitoxin (2HCl) was obtained from Dr. Harry Hines, USAMRIID, Frederick, Fort Detrick, MD.

The various probe tips and their general design are illustrated in figure 1. The tips are made to fit a VG standard continuous flow probe using either a 25 or 50 micron i.d. fused

silica capillary columns.

Sample Preparation: Two ml of human urine was amended with 20 ug of saxitoxin. A weak (pKa 4.8) cation exchange (CBA Bond-Elut,Analytichem International/Varian, Anaheim, CA) column was used for purification and was first washed with 1 ml methanol followed by 1 ml of 0.05M K_2HPO_4. The sample (2 ml urine) was loaded on a CBA Bond-Elut column after adjusting the pH value to 8 with NH_4OH. The column was washed with 5 ml water followed by 2 ml methanol. Saxitoxin was eluted with 2 ml 20% acetic acid (pH=2) and concentrated to 1 ml. The samples were injected directly into the mass spectrometer.

The solvent matrix (selected matrix) used for CF/FAB/MS was: 5 % glycerol, 0.5 % acetic acid, 0.025 % sodium dodecyl sulfate (SDS), 0.1 % polyethylene glycol (PEG) 400, 0.5 % PEG 300.

Verification of analyses was done by ionspray mass spectrometry on a Sciex Taga 6000 quadrupole mass spectrometer. The column used was a Supelco LC-18DB (1 x 300 mm) with acetonitrile and 0.1M ammonium acetate (25:75, 50 ul/min) as mobile phase.

Additional verification of STX identification was done by HPLC (Shimadzu C-R4A) using a NOVA Pak-C18 column (75 x 3.9 mm, Waters) with 10 % methanol containing 0.2 mM Na heptanesulfonate as mobile phase (0.8 ml/min). Post column oxidation was done with 1.2 N NH_4OH (0.05 ml/min) and periodic acid (0.05 ml/min)

which was added to the eluate and allowed to react for 5 min. at 55 C; 1.2 N sulfuric acid (0.06 ml/min was added for neutralization). The oxidized STX was detected by a Shimadzu (RF-530) fluorescence monitor (335 nm for excitation and 390 nm for emission).

DEVELOPMENT OF THE CF/FAB/MS METHOD

The static FAB mass spectrum of 10 ng of standard STX in thioglycerol: water (5:95) is shown in figure 2-A. The protonated molecule is found at m/z 300, thioglycerol adduct minus water at m/z 390 and the M+H minus water (m/z 282). The CF/FAB/MS of 10ng STX (standard) in 5% glycerol (selected matrix) is shown in figure 2-B. The protonated molecule (M + H, m/z 300), the M+H-H2O (m/z 282) and the methoxy derivative (m/z 314) are shown. The fragments at m/z 305, 349 and 393 are from PEG in the selected matrix although m/z 393 is also the value of (M + H + glycerol)+.The ion species at m/z 282 is a fragment ion as well as a ionization species in equilibrium with STX in solution. The methoxy derivative is not normally found and is due to methanol used somewhere in the preparation procedure; we chose to present a spectrum with the m/z 314 fragment so that readers are aware of its presence. We do not normally find the sodium adduct in our CF/FAB analyses.

We have improved the sensitivity of the CF/FAB/MS of STX through a series of progressive experiments in which the matrix,

the matrix flow rate and choice of detergent, acid, probe tip temperature and probe tip design (figure 1) were tested. In order to minimize matrix background, we chose to use a resolution of 1500 in selected ion recording (SIR) mode with m/z 300 as the selected ion.

CF/FAB matrix: The starting matrix used in our experiments with STX (5ng multiple injections) was composed of 1 % thioglycerol, .05 % acetic acid and water (figure 3-B). We improved performance by changing the matrix to 5 % thioglycerol or 5% glycerol, 1 % acetic acid and water. (figure 3-A). The 1 % thioglycerol or glycerol concentration had too low a viscosity on the probe tip and evaporated quickly whereas the 5 % concentration provided a stable base. The probe tip used in this experiment was of flat design, the injection volume was 0.5 ul and the temperature was 50 C.

Surfactant: A number of surfactants were tested for performance in CF/FAB. We compared chain length and its effect on detection and compared it with sodium dodecyl sulfate (SDS). The surfactants used were the sodium salts of 0.025 % hexanesulfonic acid, decanesulfonic acid, dodecanesulfonic acid and hexadecanesulfonic acid (figure 4-A-D). The flat probe tip was used in these experiments at a temperature of approximately 50 C. Hexanesulfonate (4-A) showed very poor performance in both sensitivity and peak shape. The sensitivity was improved with decanesulfonate (4-B) and dodecanesulfonate (4-C) but the reproducibility from injection to injection as well as peak

shape were not acceptable. Hexadecanesulfonate (data not shown) gave an extremely broad peak which was not acceptable; three replicate injections of 5 ng STX yielded no recognizable peaks while injection with 50 ng STX yielded an extremely broad peak. Addition of sodium SDS to the matrix made a dramatic effect (figure 4-D); it has properties which resulted in the best performance in terms of peak shape, sensitivity and reproducibility.

Additional experimentation revealed that 5% glycerol, 0.5% acetic acid, 0.025% SDS, 0.1% polyethylene glycol 400 (PEG) and 0.5 % PEG300 gave superior results. The PEG is used as an internal calibrant. This matrix is referred to in this text as the "selected matrix".

Probe Tip temperature and Flow Rate: The temperature of the probe tip is important in stability of the matrix because under vacuum rapid evaporation and drying can take place. It is difficult to measure the temperature exactly at the tip and only an approximation can be given. However, the peak shape is affected and hence detection at 50, 55, 60 and 70 degrees were measured (figure 5A-C). Theoretically , evaporation at the tip can be compensated for by increasing the flow rate. However, at 70 C with the "selected matrix" and a flow rate of 8 ul/min, only one of four replicate injections of STX was found (data not shown). At 50 (fig.5-A) and 60 C(fig.5-C), detection was improved but reproducibility and peak geometry was poor. Under our instrument conditions, 55 C (fig.5-B) appeared to give us

the best results although even at this temperature gradual increase of the film thickness was observed resulting in a decrease of signal with repeated injection. This temperature was used throughout the rest of our experimentation.

The input of matrix flow to the tip should be balanced with the matrix removal in order to obtain a stable thin film for an extended period (30 min) of time. The mechanism of matrix removal is exclusively governed by the evaporation rate at the probe tip, tip geometry and degree of vacuum. Using the conditions as described above, 5 and 8 ul/min flow rates were attempted using multiple injections of 500 pg STX and the flat probe tip. A flow rate of 5 ul/min (data not shown) gave poor resolution of the peaks whereas 8 ul/min were optimum for the above conditions. It is important to note that as vacuum conditions change (pumps become old or inefficient) , the flow rate will have to be adjusted. Moreover, different probe tip design may prompt an adjustment as in our case. Subsequent experimentation with fritted probe tips indicated that 5 ul/min was optimum; this flow rate was used for the rest of the experiments.

Conditioning of Probe Tip: The surface of the probe tip becomes contaminated with tar-like materials after prolonged use especially with thioglycerol. It should be cleaned with 1N HCl followed by rinsing with methanol and then the FAB matrix at 5 ul/min. for 30 minutes. These steps are necessary to insure a uniform and thin spread of the matrix over the tip surface. Our

experimentation has shown that the unconditioned tip surface can initially produce a very thin liquid film and give very high sensitivity but is unstable and soon collapses. The conditioned tip produces a thicker but stable film which is less sensitive but more reproducible. Components with a high boiling point will accumulate at the tip disrupting the dynamic equilibrium and causing memory effects. Moreover, as the volume of the fluid at the tip increases , the sensitivity decreases because dilution and tailing increases.

Selection of Probe Tip: We examined how the shape of the probe tip affects peak shape, reproducibility and sensitivity. We compared the flat, tapered and round probe tips versus the fritted(figure 1). The "selected matrix", probe temperature of 55 C, flow rate of 5 ul/ minute, 50 micron i.d. open bore fused silica capillary column (deactivated), 500 picogram STX and a 0.5 ul injection volume was used in these experiments. Comparison of the flat (fig.6C), round (fig.6A) and tapered (fig.6B) tips indicated that the flat surfaced tip gave the best results based on the above criteria.

The poor performance and shape of the peaks obtained from the round tip (figure 6-A) is due to the fact that the tip dries quickly in the center because of the small surface area . The tapered tip (figure 6-B) does not allow smooth and even film flow on the surface as we expected; rather it forms an unstable thick droplet which falls off at various intervals. Of the three, the flat tip appears to have the correct flow

characteristics (figure 6-C) with a peak width at the base of approximately 30-35 seconds. We found that 5-8 ul/min.(depending on the tip temperature) gave optimum results although others use 1 ul/min.as their optimum; this may be due to the temperature at the tip surface.

Three types of fritted/wick-type (Wonjo, VG Woolfitt and Olson/Hogge) CF/FAB probe tips were compared for performance characteristics as described above for saxitoxin using the same conditions. The Wonjo-tip (figure 7A) was designed in our laboratory as part of the method development effort. Analysis using the Wonjo tip and multiple injections of 300 pg STX gave satisfactory results (peak width at base 30-35 seconds) in terms of sensitivity except that with repeated injections (60 nl Rheodyne injector) the fine mesh stainless steel screen in the tip tended to plug until no signal was detected. Cleaning was tedious and often the screen was lost.

The VG Analytical (Woolfitt design) was tested with multiple injections of 300 picograms of STX for comparison (figure 7B). The reproducibility and sensitivity was superior to the Wonjo tip but it also tended to plug with the particular matrix and mass marker(PEG 300 and PEG 400) used. The wick provided a large advantage in flow characteristics because it prevented droplet formation which resulted in loss of signal. The peak width at the base was 30-35 seconds (injection volume of 60 nl and 5 ul/min flow rate).

The most dependable performance was obtained with the Olson/Hogge probe tip developed in the laboratory of Dr. Lawrence Hogge, NRC, Saskatoon, Sask. Canada (figure 8). The peak width at the base of 500 pg of STX (60 nl injection volume) was 30 seconds and that with the 200 pg injection was 10-12 seconds; the reproducibility was excellent. Although the design of the latter is much like that of the VG Analytical (Woolfitt) tip, the performance for STX is superior in our hands and is the one we recommend for STX. The primary determinant for the performance of any continuous flow probe tip design is geometry and peak width. The Olson/Hogge design satisfied this requirement; it is the only tip that gave us such narrow peak geometry for STX. Other compounds may give different results in terms of peak shape but we use those obtained with STX and the Olson/Hogge tip as a standard.

Analysis of STX from human urine: The urine sample was amended with STX , extracted, purified through a CBA column as described and made ready for injection (in acetonitrile) into the mass spectrometer. The CF/FAB/MS analyses (multiple, 200 nl open-loop injections) are shown in figure 9. The peak shape profile shown is that of m/z 300 (M + H)of the total ion current ; the scan range was from m/z 250 to 350. The injections shown are those of STX spiked urine as well as the column rinse as indicated. The peak width at the base was 25 seconds. The urine sample was spiked with STX at a level of 20 ppm and the percent recovery was 75%. The identity of the STX in the urine was verified by both HPLC (C18 column) and Ionspray MS (Supelco LC

18 DB column).

The mass spectral scans of the constituents coeluting with STX in the CF/FAB/MS are shown in figure 10. STX fragments are found at m/z 282 (M+H minus 18) and 300 (M + H). Occasionally m/z 314 (methoxy derivative of STX) is found. The composition of the urine blank is shown in figure 10-B. The major urine components are found at m/z 287 and 311. The ions at m/z 261, 305 and 349 are from PEG calibrant. Quilliam (26) reported both m/z 314 and 328 (two methoxy groups) when using methanol in ionspray mass spectrometry; both were eliminated when acetonitrile was used. We found both fragments in some but all of our experiments except that m/z 314 was encountered more frequently.

CONCLUSIONS

We have developed an improved CF/FAB/MS technique for the analysis of STX in urine where 200 picograms can be detected using an open bore fused silica column (i.d. 50 um). Addition of a small amount of surfactant (Na dodecylsulfate) facilitates solubility of STX in the FAB matrix so that more of the STX molecules are at the surface of the matrix. The average penetration of the xenon beam into the matrix is about 100 angstroms. The use of 50 um i.d. capillary columns allows the detection of a rather distinct peak band which contains the analyte in question as well other coeluting compounds; 25 um i.d. give sharper peak bands. Although chromatographic resolution is not obtained, STX can be detected from among the

other components through single ion chromatograms. The latter is an efficient method of detection because it precludes the use of chromatographic columns which often require solvent systems and flow rates not compatible with FAB. HPLC solvent systems are not compatible with the FAB matrix and cause loss of signal i.e. signal quenching. Some of this can be overcome with the use of coaxial tees which allow separate introduction of HPLC solvent and FAB matrix but also complicates the analytical procedure.

Although the CF/FAB/MS urine analysis shows promise, its sensitivity into the parts per billion range needs to be perfected. Moreover, the information learned in this study will allow us to look for and detect metabolic derivatives of STX in the urine of rats dosed with STX. Although a comparison of this method with detection by ionspray mass spectrometry was not intended, it is encouraging to learn that in our laboratory and under our conditions, CF/FAB in SIR allows detection of at least 100-200 pg as compared with 10-15 pg in ionspray.

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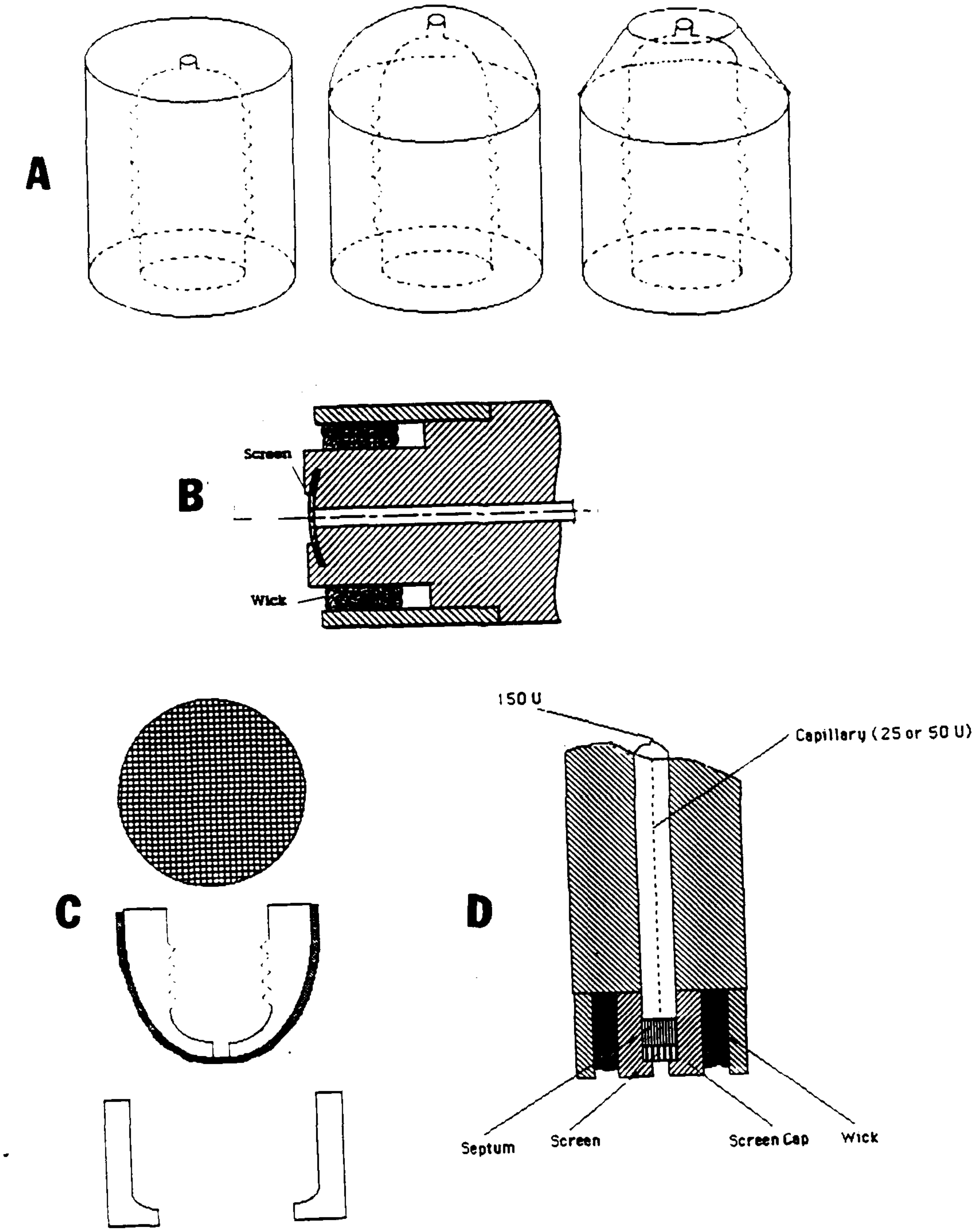

Figure 1A-D. General design of the various FAB probe tips used in this study. (A) General representation of the standard VG probe tip modified in design as flat, oval and beveled. (B) The VG frit/wick tip as designed by Dr. Adrian Woolfitt of Fisons Instruments. (C) The Wonjo tip made up of a fine mesh stainless steel screen which fits around the tip secured in place by friction with a metal cap.(D) The Olson/Hogge tip complete with screen, septum,screen cap and wick. In all cases the probe tips are made to accommodate capillary columns 150 microns in diameter and an i.d. of 25 or 50 microns.

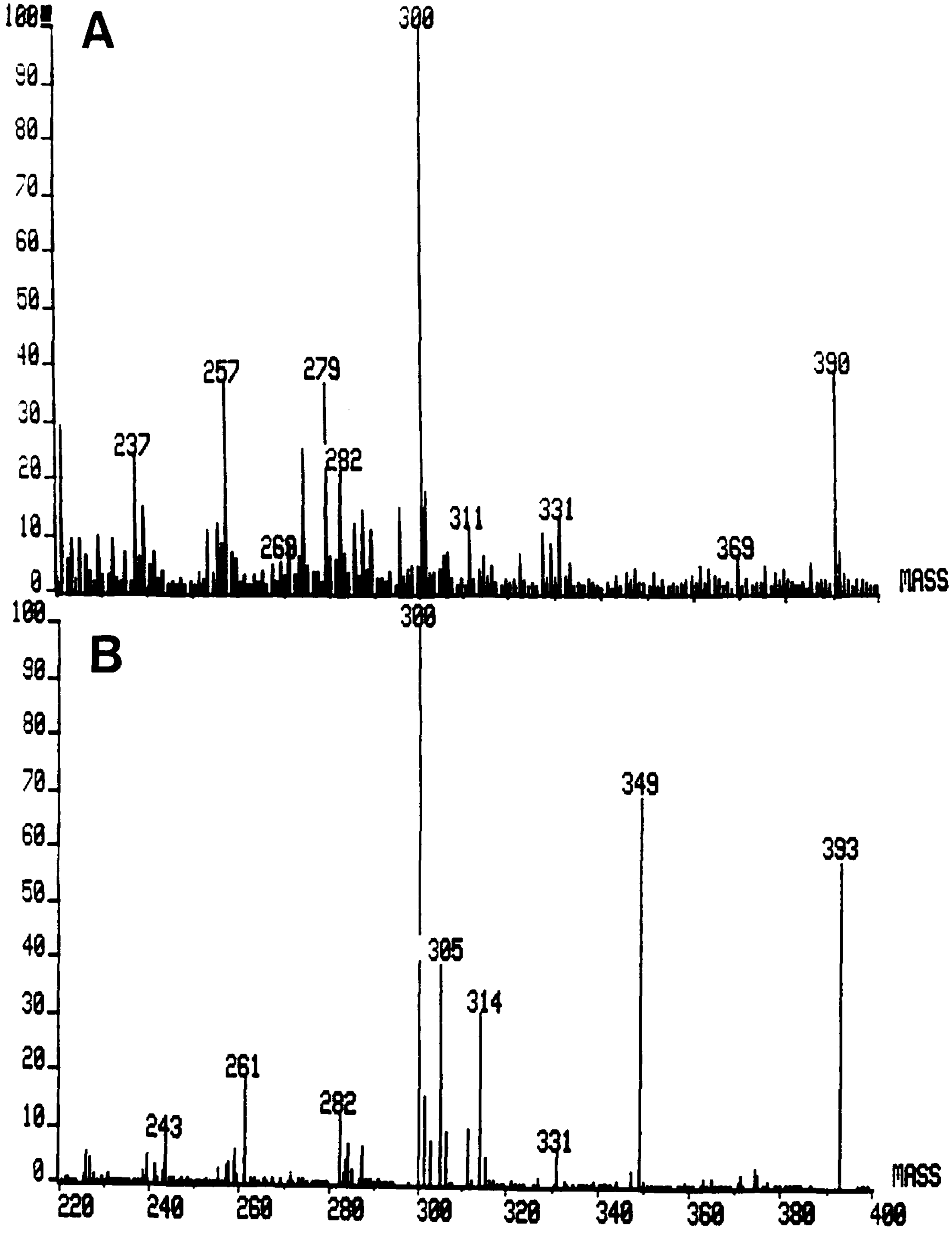

Figure 2A-B. (A) Static FAB/MS of a standard of STX (10 ng) in thioglycerol:water (95:5). The protonated molecule is found at m/z 300, the thioglycerol adduct minus water at m/z 390 and the dehydrated M+H at 282. (B) CF/FAB mass spectrum (average of 20 spectra) of 10 ng saxitoxin in glycerol (selected matrix) and a flow rate of 8 ul/min. The protonated molecular ion is found at m/z 300, the dehydrated molecular ion at 282 and the methoxy derivative at 314. The fragments at m/z 305, 349 and 393 are from PEG in the matrix.

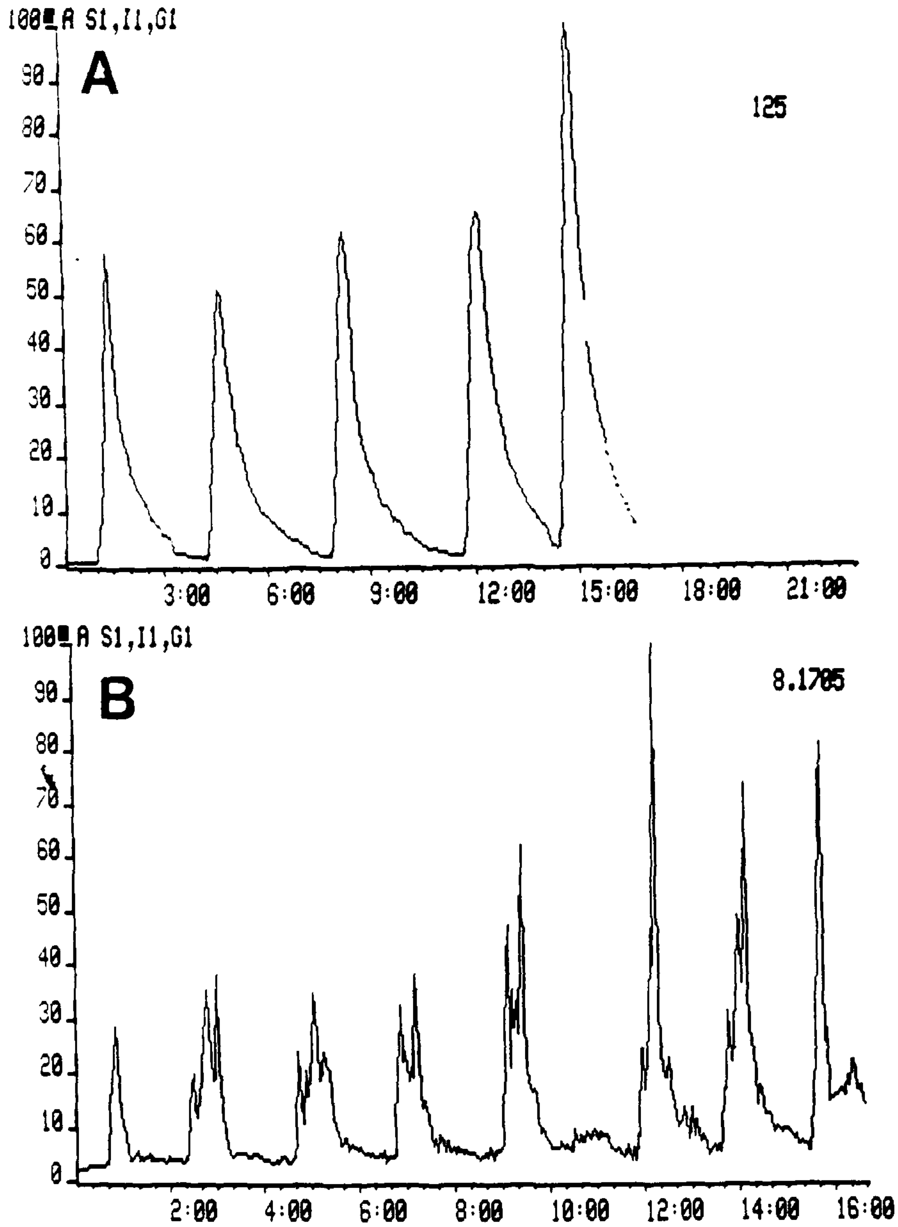

Figure 3A-B. Effect of concentration of thioglycerol in the FAB matrix on sensitivity and peak shape of STX using CF/FAB and SIR (probe tip at ~50 C) at m/z 300, resolution of 1500, 0.5 ul injection volume and a flat probe tip.(A) Multiple injections of 5 ng STX using a 5 % thioglycerol and 0.05 % acetic acid. (B). Same injection as in A but using 1 % thioglycerol.

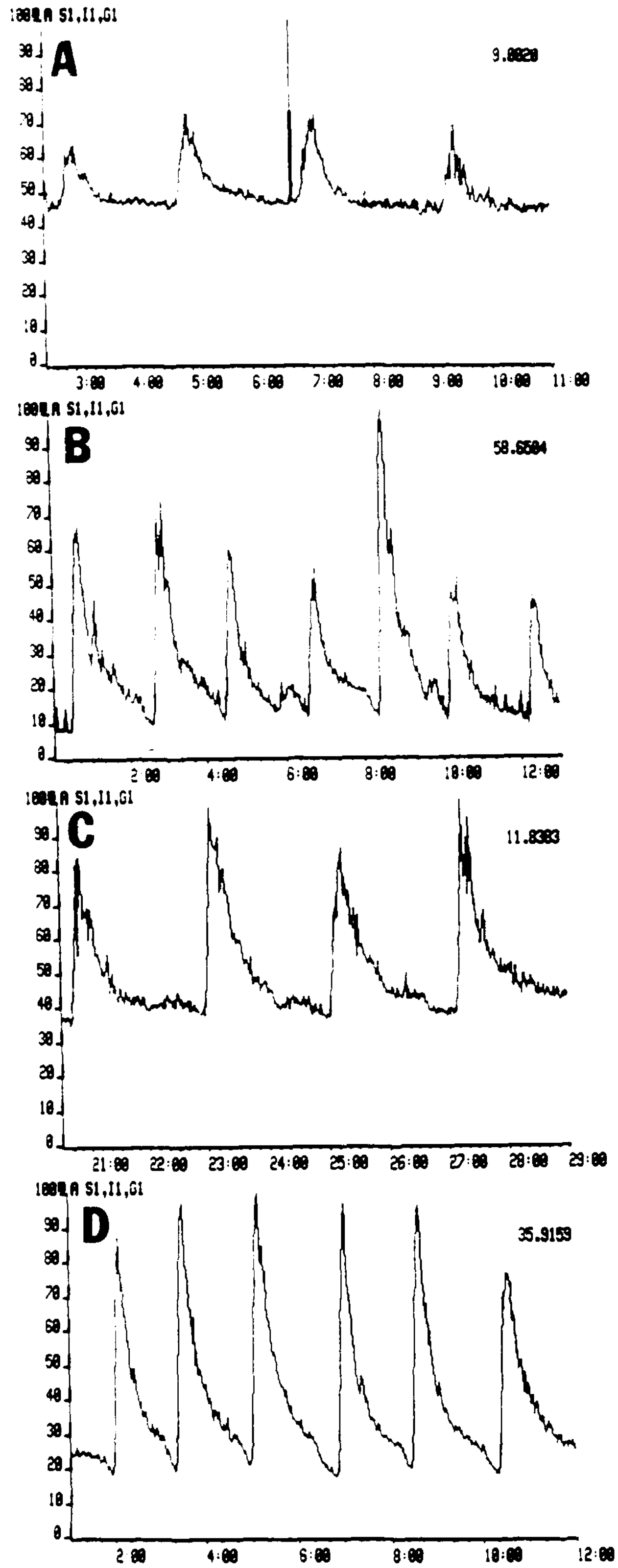

Figure 4A-D. Effect of surfactant on the detection of STX (5 ng) by CF/FAB/MS in SIR at m/z 300 using the "selected matrix" in which only the surfactant was varied. The flat probe tip was used with an injection volume of 0.5 ul at 50 C. (A). 0.025% Na hexanesulfonate (B). 0.025% Na decanesulfonate (C). 0.025% Na dodecanesulfonate (D). 0.025% Na dodecylsulfate.

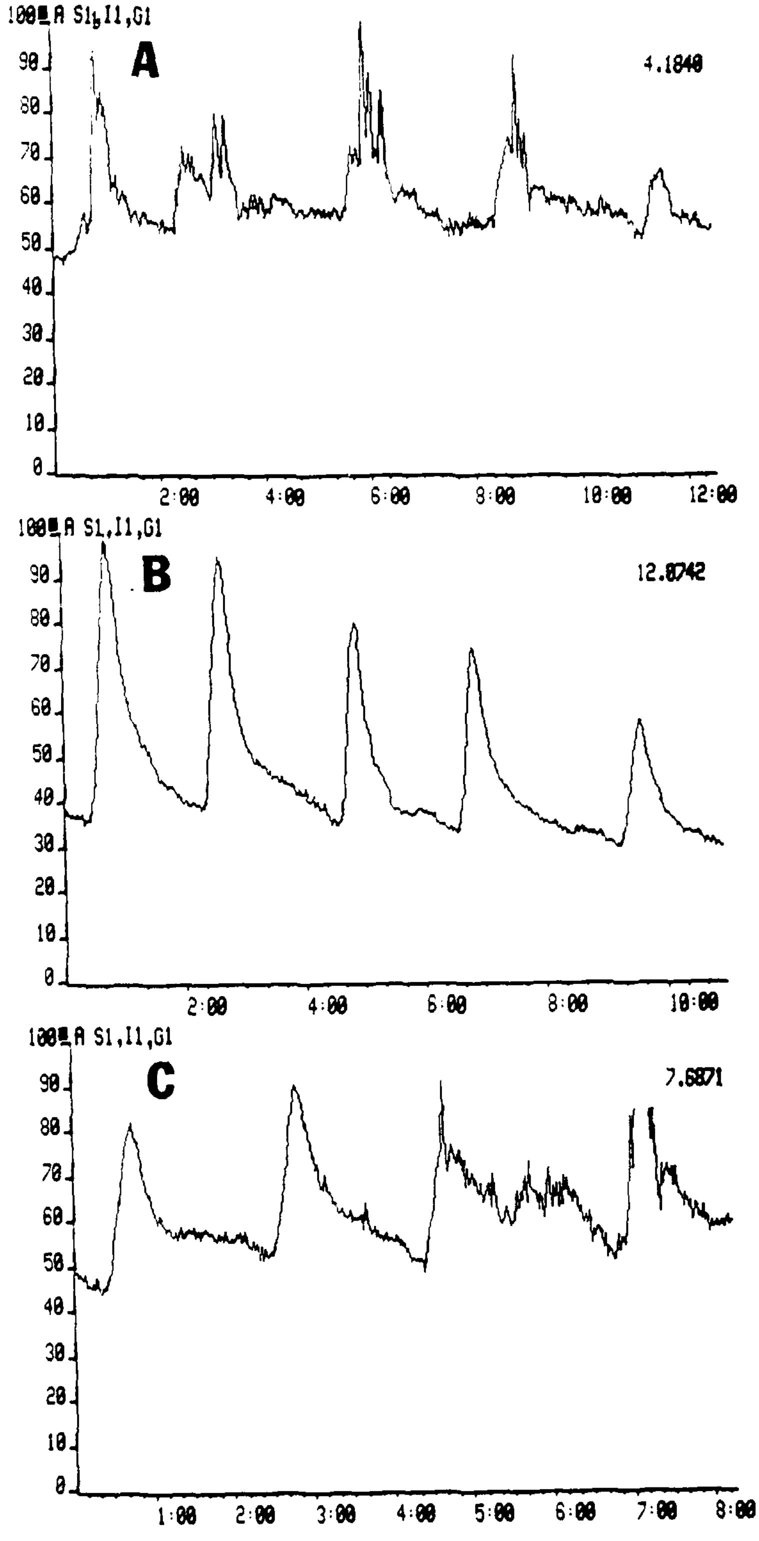

Figure 5A-C. Effect of tip temperature on analysis of STX by CF/FAB MS. Multiple injections (500 pg) of STX were made using the "selected matrix" and flow rate of 8 ul/min. (A) 50 C; (B) 55 C; and (C) 60 C.

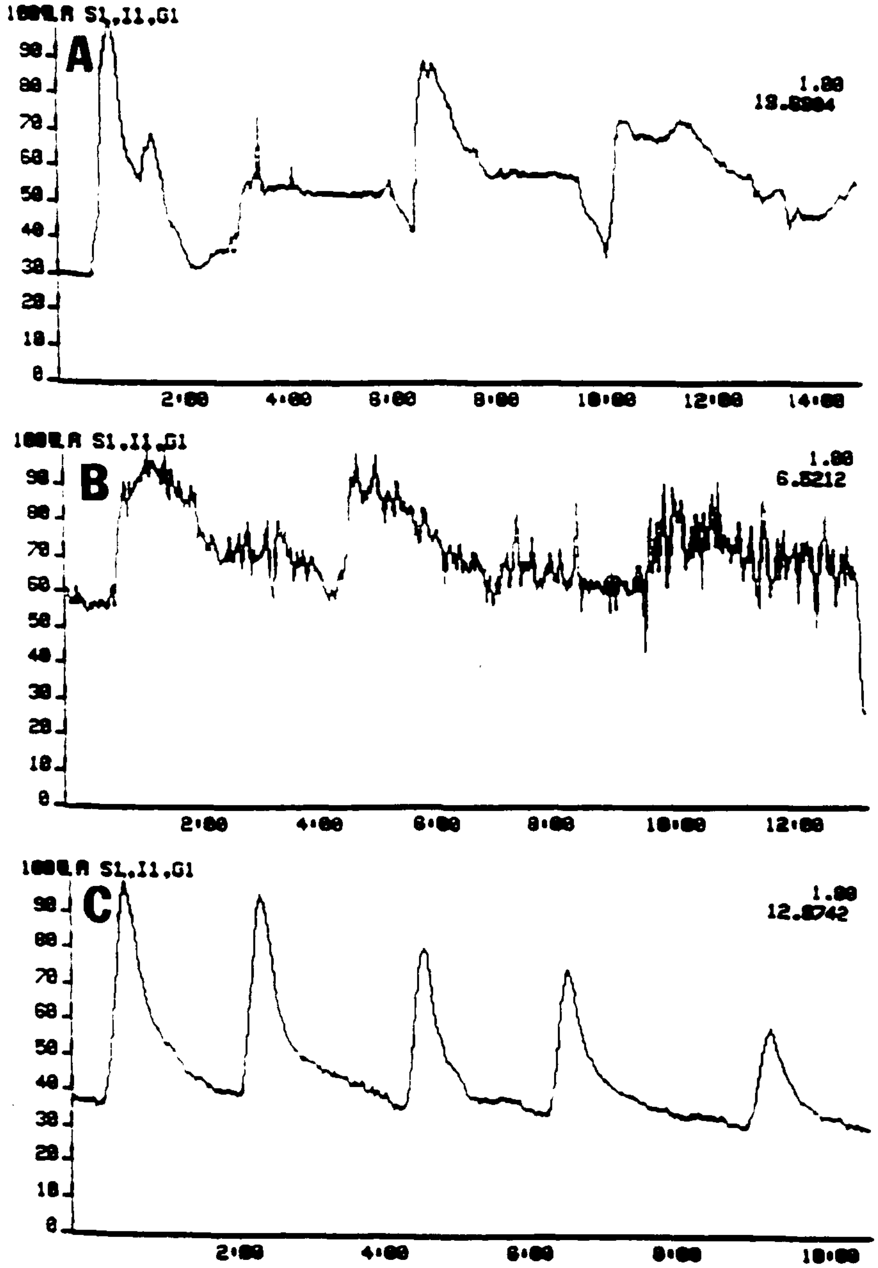

Figure 6A-C). Effect of probe tip geometry on detection, sensitivity and peak shape of saxitoxin. Multiple injections of 500 pg STX, "selected matrix" , 55 C probe tip temperature ,0.5 ul injection volume, 50 micron i.d. open bore fused silica capillary column were used and a flow rate of 5 ul/min. (A) round probe tip (B) tapered probe tip and (C) flat tip probe; the peak width at the base is about 40-50 seconds.

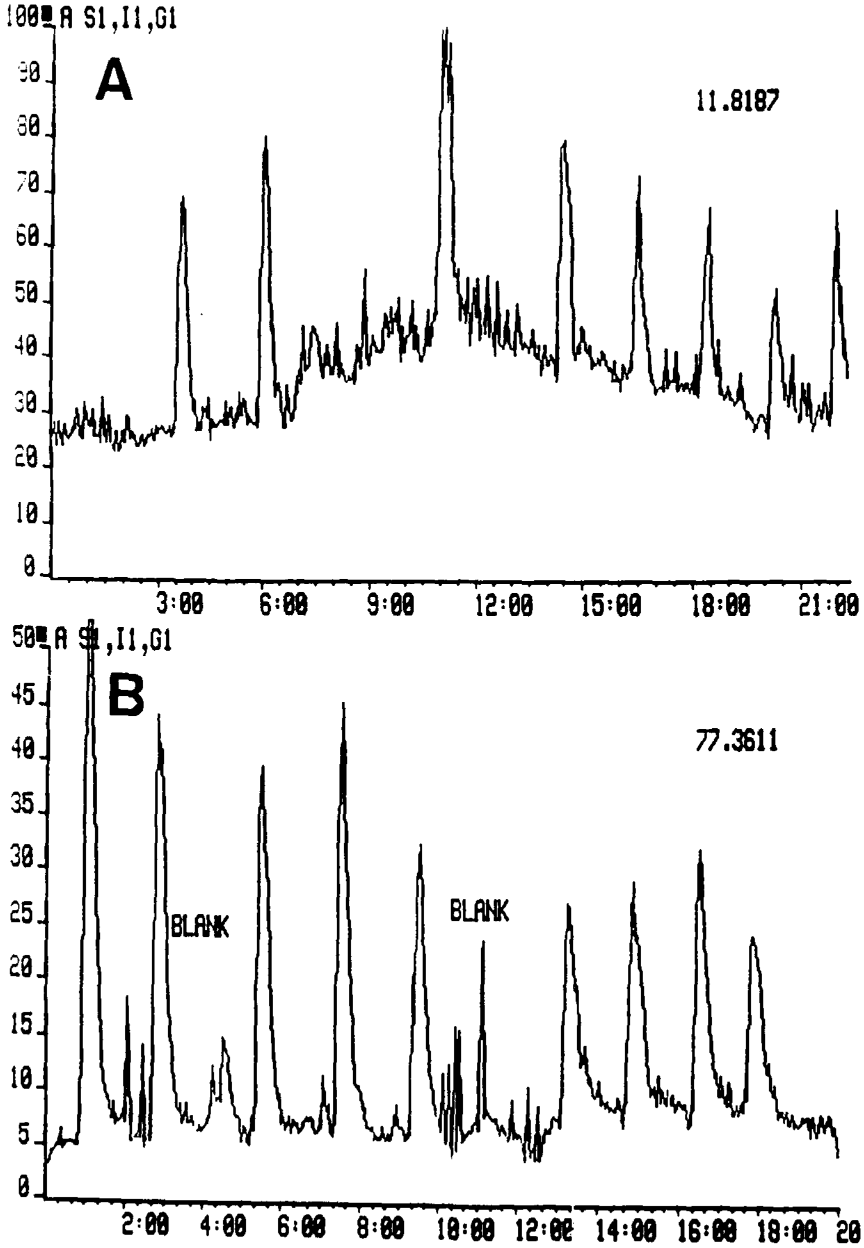

Figure 7A-B. (A) Multiple injection of 300 picograms (60 nl injection volume) of STX using the Wonjo probe tip in CF/FAB/MS. Analytical conditions were: "selected matrix", 5 ul/ min., 55 C probe tip temp, 50 um i.d. open bore fused silica capillary column. The probe consisted of a stainless steel sleeve which fitted over an oval nose cone which in turn held a stainless steel fine mesh screen. (B) Multiple injection of 300 picograms (same conditions as in A) of STX using the VG Analytical/Woolfitt probe tip in CF/FAB/MS.

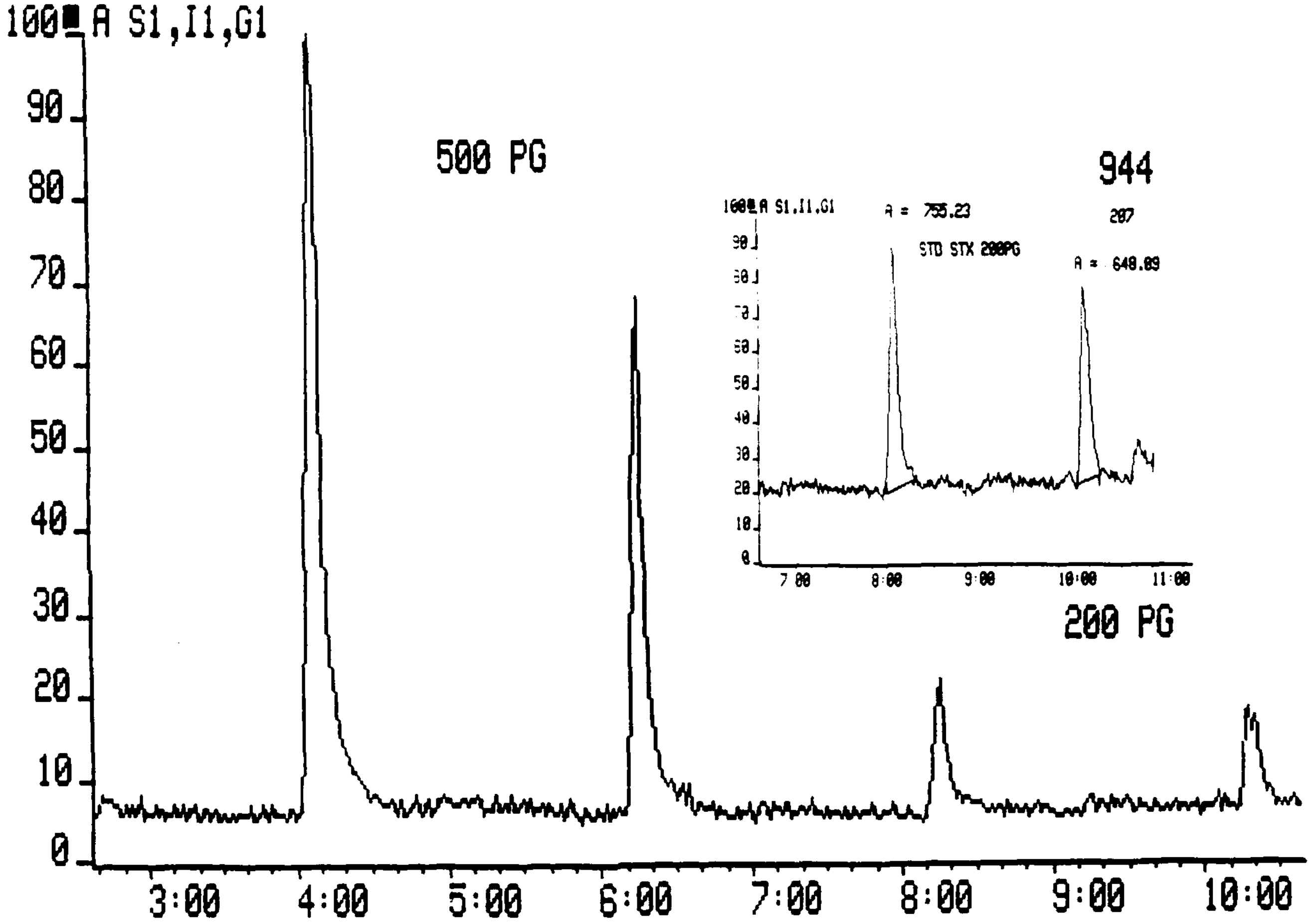

Figure 8. Multiple injections of 500 and 200 picograms respectively of STX using the Olson/Hogge probe tip in CF/FAB/MS. The FAB conditions were the same as described in figure 7. The peak width at the base of the 500 pg injection is about 30 sec. and that of the 200 pg peak is about 10-12 seconds. The inset shows the peak shape, area and signal to noise obtained with 2 consecutive injections of 200 pg STX.

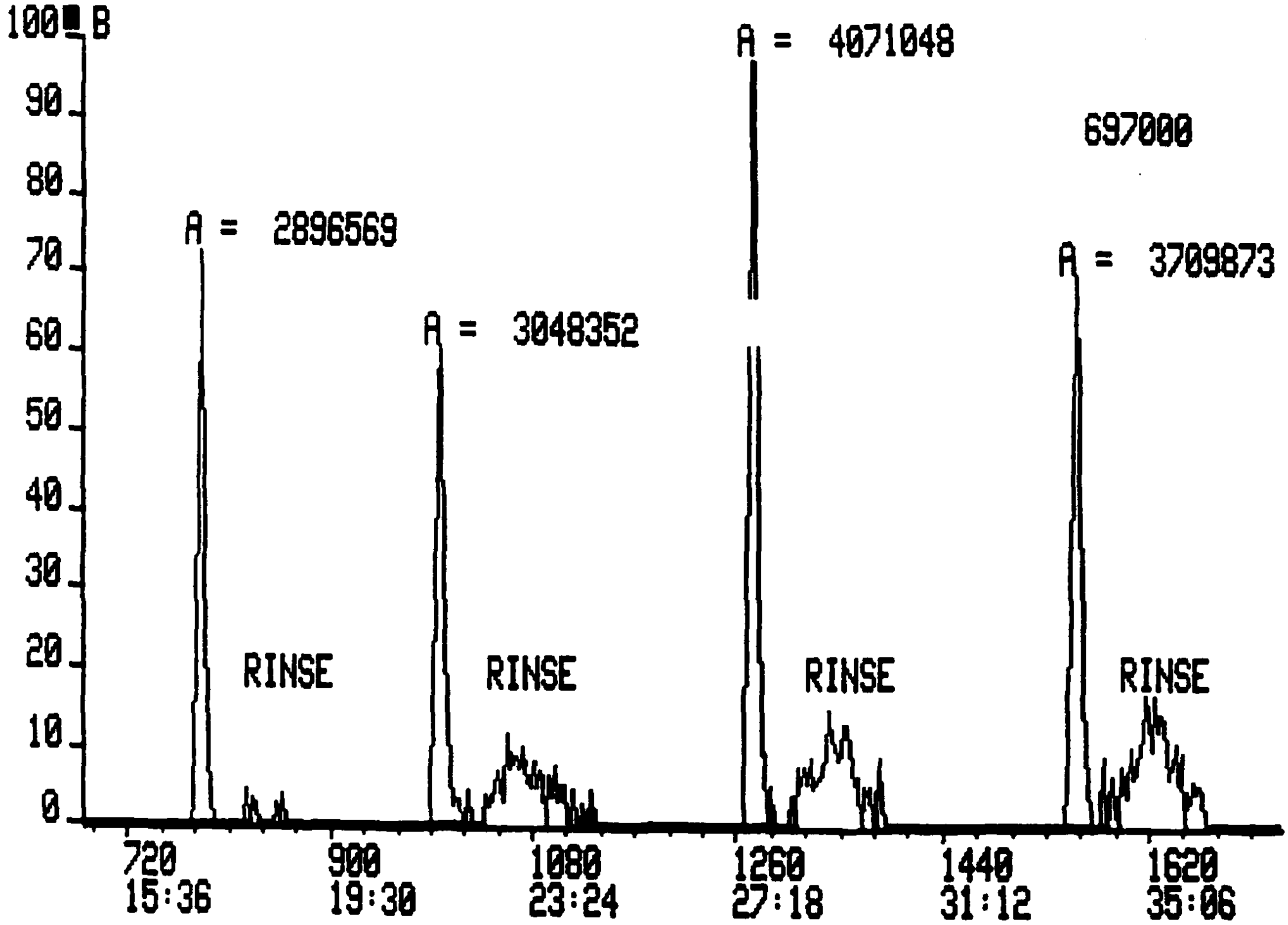

Figure 9. CF/FAB/MS (Olson/Hogge tip) analysis (multiple injection) for STX extracted from human urine, purified on a CBA column, 50 um i.d. open tubular deactivated capillary column, 200 nl injection volume, 5 ul/min. flow rate and 55 C probe temperature. Each of the four replicate sample injection peaks (m/z 300 of the total ion current scanned between m/z 250-350) is followed by a solvent rinse. The peak width average at the base is approximately 25 seconds and the average area of the four peaks is 3,431,460.

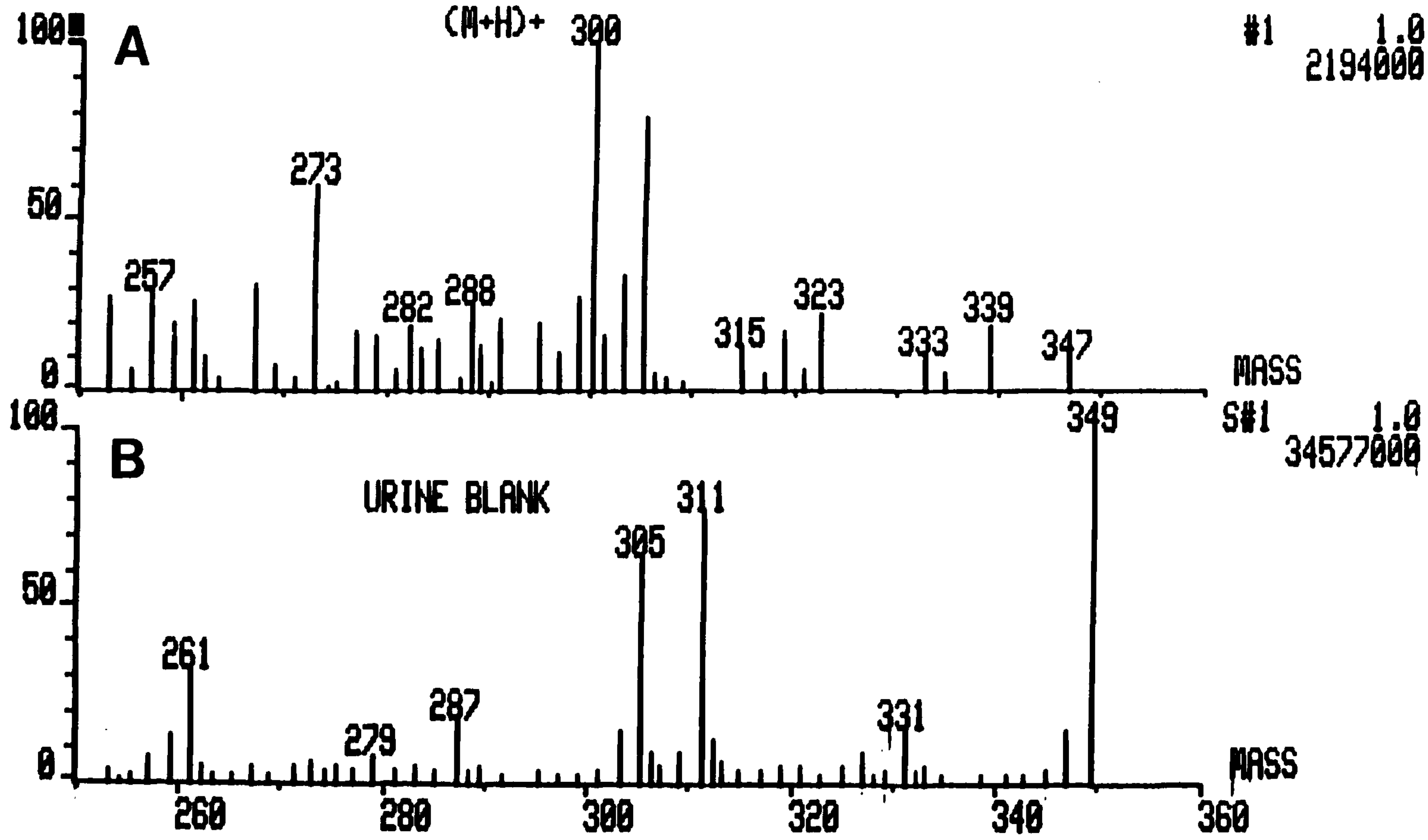

Figure 10. CF/FAB mass spectrum of the components found in the sample of STX spiked urine. Refer to peak #2 (scan 999) of figure 9. (B) CF/FAB MS of urine control sample. Fragments with m/z values of 287 and 311 are urine components. Fragments at m/z 261, 305 and 349 are from the PEG calibrant.